Effect of Growth Regulators on Micropropagation of Japanese Orange

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Abstract:

The study was conducted in the Plant Tissue Culture Laboratory of the Department of Horticulture and Landscape gardening, College of Agriculture, Al-Qasim Green University, during the period from November 2022 to June 2023 as a factorial experiment using a completely randomized design (CRD) with two factors and ten replicates, the first factor being Putrescine (PUT) at four concentrations (0 , 2.5, 5 and 7.5 mg L^{-1}) and the second factor (CPPU) at three concentrations (0, 4 and 8 mg L^{-1}) added to the nutrient medium to determine their effect on the growth of multiplying kumquat (Japanese orange) plants in vitro. The results can be summarized as follows:

1- The results showed that there were significant differences between the concentrations of Putrescine (PUT) added to the nutrient medium in most of the vegetative and chemical growth traits at the concentration (7.5 mg L^{-1}), which was significantly excelled on the other concentrations as it recorded the highest average in branch length, number of leaves, and fresh weight. And the survival of the crops and the content of the shoots of chlorophyll and carotenoids, and the content of the shoots of total soluble sugars, nitrogen, phosphorus, potassium, auxin, and cytokinins (2.0011 mg 100 g^{-1}, 1.9267 mg 100 g^{-1}, 10.0956 mg g^{-1}, 13.5811 µg kg. $^{-1}$, 24.2344 µg kg^{-1}) respectively, compared to the lowest results with the control treatment (0)While the concentration (5 mg L^{-1}) showed a significantly excelled on the other concentrations, as it recorded the highest rates in gibberellin, reaching (31.820 µg kg^{-1}), respectively, compared to the control treatment (0).

2- The results showed that there were significant differences between the concentrations of CPPU added to the nutrient medium in most of the vegetative growth traits and chemical traits, especially at the concentration (0.8 mg L^{-1}), which was significantly excelled on the other concentrations as it recorded the highest rates in the shoots' content of chlorophyll and carotenoids. The content of the shoots of total soluble sugars, auxin, and cytokinins reached (2.1383 mg.$100g^{-1}$, 1.9908 mg.$100g^{-1}$, 10.1792 mg.L^{-1}, 13.7992 µg kg^{-1}, 24.4975 µg kg^{-1}), respectively compared to By transaction (0). Adding a concentration (0.4 mg L-1) showed significantly excelled on the other concentrations, as the highest rate was recorded in gibberellin, reaching (32.249 µg kg^{-1}) compared to the control treatment (0)The bi- interaction between the two experimental factors, (PUT) and CPPU, had a similar significant effect on most of the studied traits, especially with the interaction treatments. There was a

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significant difference for the interaction (PUTx0.8CPPU7.5) in its chlorophyll content, the carotene content of the shoots, and the shoots' content of Sugars, the auxin content of the shoots, and the shoots' content of cytokinins were recorded (2.5133 mg 100 g^{-1}, 2.3367 mg 100 g^{-1}, 10.5700 mg L^{-1}, 14.3633 µg kg-1, 25.3733 µg kg^{-1}) respectively compared with the interaction (PUT 0 x CPPU 0)

Keywords: Plant Tissue Culture, Pyotrcine (PUT), CPPU, NAA, BA

Introduction:

The round Kumquat Marumi Kumquat, which belongs to the Rutaceae family, is one of the species of the genus Fortunella, whose scientific name is "Fortunella Japonica". It is native to southeastern China. Its shape resembles an orange, and its size is small, the size of a large olive. Hundreds of these fruits grow on small shrubs. Kumquat fruit is considered one of the favorite fruits for making jam, jelly candy and many types of sweets, due to its sweet and sour taste. It is suitable for being ornamental plants as it has few or almost no thorns and is slow growing. Citrus trees take an important place among fruit trees due to their importance. Nutritional, economic, medical, aesthetic and environmental, as its fruits are rich in mineral salts necessary for building the human body such as potassium, calcium, iron, magnesium, sodium, sulfur and phosphorus. They are also a source of vitamin C [5]. Propagation of citrus by traditional methods is limited to a specific season, in addition to the availability of explant. Also, these methods do not guarantee the production of a large quantity of citrus identical to the variety throughout the year, so the technique of plant tissue culture appeared to propagate and improve many woody plants, including citrus [12] .

Plant growth regulators play a major role in many important physiological activities and are also involved in regulating the growth of the plants. One of these regulators is CPPU, which is one of the highly effective synthetic cytokines that is 10 times more effective than benzyl adenine (BA) when it works to break apical dominance. And stimulating the growth of lateral shoots [14]. PUT (Putrescine) and its plant formula (H12N2.2HclC4) is one of the types of the group of polyamines and is the primary source for the formation of other types (spermidine and permin). It is an organic compound, and is considered the lowest molecular weight of the amines, which gives it rapid transfer between cell components or plant organs [4] as it was found to play an important role in cell division, flowering processes and increasing plants' tolerance to environmental conditions [15].The study aims to determine the extent of the effect of the growth regulators Putrescine and CPPU and their interaction on the growth and multiplication of Kumquat (Japanese orange) shoots in vitro.

Materials and methods:

The study was conducted in the Plant Tissue Culture Laboratory of the Department of

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Horticulture and Landscape Engineering - College of Agriculture, Al-Qasim Green University, during the period from November 2022 AD to June 2023 AD. To evaluate and study the effect of the growth regulators CPPU and PUT on the growth and multiplication of Kumquat (Japanese orange) shoots in vitro. The nutrient medium approved by [3], known by the abbreviation MS salts, was used, produced by the American company Callsion, and the weight was 4.43 g/L according to the recommended instructions. In order to micropropagate the plant, 3% sucrose and myo-inositol were added to it. (Myo-inositol) 100 mg/L and 2 mg/L BA (An amount of 100 mg of BA was weighted and dissolved in drops of absolute ethanol and the volume was completed to 100 ml. Take 2 ml from this solution and add to a liter of medium if desired to prepare a liter of medium It contains 2 mg) and 0.1 mg/L NAA (an amount of 100 mg of NAA was weighted and dissolved in drops of absolute ethanol and the volume was brought to 100 ml. Take 0.1 ml from this solution and add to a liter of medium if desired to prepare a liter of medium containing 0. 1 mg), and 7 g agar (Agar-Agar type) was added to it, then the pH of the medium was adjusted to 5.7 ± 0.1 with 1 standard solution of hydrochloric acid (HCl) or 1 standard solution of sodium hydroxide (NaOH), and the cultures were incubated in the growth chamber on temperature 25±º2 C and lighting intensity 1000 lux for 16 hours/day for four weeks.

Chemical measurements included:

1- Total chlorophyll content of shoots (mg.100g^{-1} fresh weight).

Total chlorophyll in shoots was estimated according to what was stated by Goodwin (1976) by taking 100 mg of the fresh sample of shoots, adding 20 ml of 85% acetone, crushing the tissue with a ceramic mortar twice, then isolating the dye solution from the plant tissue using filter paper. The filtrate was taken, leaving a precipitate, and then the volume was completed to 20 ml of acetone.

A UV-visible Spectrophotometer was used in the Graduate Studies Laboratory at the College of Agriculture at Al-Qasim Green University, Department of Horticulture and Landscape Engineering, to measure the optical absorption of total chlorophyll pigment at two wavelengths, 645 and 663 nm. Then the amount of pigment was calculated (mg pigment/100 g fresh plant tissue) by applying the following equation :

Total chlorophyll=20.2 x D (645) +8.02 x D (663) (V/W x1000) x 100

So: D (645) = Optical absorption reading at a wavelength of 645 nm

D (663) = Optical absorption reading at a wavelength of 663 nm

V = final volume of extract (20 ml)

W = weight of plant tissue (1g)2 - Estimation of the carotenoid content of branches (mg.100 g^{-1} fresh weight)

Carotene was estimated in the same way as mentioned above in determining the total

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chlorophyll pigment. The optical density was read with the same device at a wavelength of 480 nm, and the total amount of carotenoids was calculated according to the following equation:

Carotene content of the sample (mg.100 g^{-1} fresh weight) = Optical density at wavelength 480.

3 - Total soluble sugars content of shoots (mg.g-1 dry weight)

The total soluble sugar content of shoots was determined by the phenol-sulfuric acid method according to the method proposed by Herbert and others (1971).

The soluble sugars were extracted by taking 100 mg of dry weight from shoots exposed to different levels of PEG and Se. They were placed in a ceramic jar and 10 ml of ion-free distilled water was added to it. After homogenizing the mixture, it was centrifuged centrally in a centrifugal device at a speed of 1500 rpm for 10 minutes, then take the filtrated and supplement its volume to 10 ml with distilled water free of ions.

Take 1 ml of it and add 1 ml of phenol reagent 5% (w/v) and 5 ml of concentrated sulfuric acid, mix well and incubate in a water bath at a temperature of 25-35 °C for 30 minutes. Then the dissolved sugars were estimated by measuring the intensity of the resulting color using Spectrophotometer at wavelength 448.

4 - Measuring the plant hormone content of branches (µg/kg fresh weight)

Gibberellins (GA3), cytokinins (Kinetin), auxins (IAA), and abscisic acid (ABA) were extracted and estimated by using a high-performance liquid chromatography (HPLC) device, by taking 5 grams of branches growing in nutrient medium supplied with polyethylene glycol and for all roots used in the study. These parts were cut individually with a surgical blade into small pieces, then frozen in the freezer and then placed in a solution of 80% Ethanol for 48 hours at a temperature of 5°C. The extract was then filtered using filter paper under vacuum. To speed up the filtration process, the filtrate was placed in a rotary evaporator under vacuum at a temperature of 30°C until the volume reached 20% of the original volume of the applied material. Then the volume was completed to 60 ml by adding deionized distilled water, then it was added to The solution is 8 ml of basic lead acetate at a concentration of 40% and drops of potassium oxalate at a concentration of 22% for the purpose of obtaining a clear solution, then centrifuging it using a centrifuge for 12 minutes and at a speed of 3000 rpm, then taking the liquid and completing the volume to 60 ml with water. It was devoid of ions and was divided into two equal parts: In the first, the hydrogen function number (pH) was adjusted to 2.5 using hydrochloric acid (double standard HCl) and then washed three times in a row in separating funnels by adding equal volumes of diethyl ether while neglecting the aqueous phase. The remaining phase of the

ISSN 2072-3857

diethyl ether was then taken to the rotary evaporator and evaporated at a temperature of 30°C until dry. Then the materials were re-dissolved by adding 15 ml of absolute methyl alcohol, and transferred to the rotary evaporator to be concentrated at a temperature of 45°C. To about 5 ml, it was kept in the freezer until the auxins, gibberellins, and abscisic acid were determined.

As for the second part of the clear solution, its pH was set to 8.6 by using a 1% NaOH solution and was washed three times in a row using equal volumes of ethyl acetate. The aqueous phase was neglected as we mentioned above, while keeping the basic ethyl acetate, which in turn It was evaporated in the rotary evaporator, after which 15 ml of absolute methyl alcohol was added and then transferred to the rotary evaporator to concentrate to 5 ml. It was kept in the freezer for the purpose of determining cytokines using the HPLC device equipped from Shimadzu Company, and 20 microliters of the sample was injected into the reversed-phase column using an injector. Injector type Rheodyn-712. The device was handled using a CR-4A calculator . Standard solutions of GA3, ABA, IAA, and Kinetin were prepared, ranging from (0.1-2.5 mg/L) by injecting different concentrations of the basic solution through a series of dilutions so that Concentrations less than 0.1 µg/L can be measured by concentrating the extraction solution or increasing the volume of the sample. The retention time, the peak area, and the height of the bands resulting from sample injections were measured and compared with the bands obtained with the bands of the standard solution resulting under the same conditions, the concentrations of plant hormones were calculated using the following equation:

Concentration (µg/kg fresh weight)=$\frac{sample\ area}{Model\ area}$× Standard model concentration

Experimental design and statistical analysis

The study was implemented as a factorial experiment using a completely randomized design (CRD) [2] and with two factors (4 concentrations of PUTx 3 concentrations of CPPU) with 10 replications. The ready-made statistical analysis system Genstat was used under the windows computer operating system to conduct statistical analyses. The averages of the coefficients were compared using the Least Significant Differences (L.S.D) test at the probability level of 0.05 to test the significant differences between the averages.

Results and discussion

1- *Total chlorophyll content of shoots (mg.100 g^{-1} fresh weight)*

The results presented in Table 1 show that there is a significant effect on the chlorophyll content of shoots. The concentration of 7.5 mg L^{-1} gave the highest rate, amounting to 2.0011 mg.100 gm-1 fresh weight, while the 2.5 mg L^{-1} treatment gave the lowest rate, amounting to 1.7522 mg.100 gm^{-1} fresh weight.

ISSN 2072-3857

Table 1 indicates the significant effect of CPPU, as the concentration of 0.8 mg L^{-1} was significantly excelled on the highest rate for the studied trait, which amounted to 2.1383 mg.100 g^{-1} fresh weight. While the control concentration of 0 mg L^{-1} gave the lowest rate of 1.4325 mg.100 g^{-1} fresh weight. As for the interaction between the two experimental factors, it had a significant effect on the chlorophyll content of shoots, as the interaction treatment (0.8 CPPU × 7.5 PUT.) was significantly excelled on all other treatments. It gave the highest rate of 2.5133 mg.100 g^{-1} fresh weight, compared to interaction treatment (PUT 0 x CPPU 0), which recorded the lowest rate of 1.1300 mg.100 g^{-1} fresh weight.

Table (1): The effect of the growth regulators PUT and CPPU and their interaction on the average chlorophyll content of shoots. 100 mg-1 fresh weight of kumquat plants after four weeks of culture in vitro.

average CPPU	PUT concentrations (mg.L-1)				CPPU concentrations mg.L-1
	7.5	5	2.5	0	
1.4325	1.2233	1.8900	1.4867	1.1300	0
1.8975	2.2667	1.3867	2.1733	1.7633	0.4
2.1383	2.5133	2.0233	1.5967	2.4200	0.8
	2.0011	1.7667	1.7522	1.7711	PUT average
0.02282= PUT 0.01976= CPPU 0.03952 = interaction					LSD (0.05)

2- Carotene content of shoots (mg.100 g^{-1} fresh weight)

The results of the statistical analysis presented in Table 2 indicate that there are significant differences between the concentrations of PUT in the carotene content of the leaves, where the concentration of 7.5 mg L^{-1} excelled on the rest of the concentrations by giving it the highest rate of 1.9267 mg.100 gm^{-1} fresh weight.While the concentration of 0 mg L^{-1} gave the lowest rate of 1.6989 mg.100 g^{-1} fresh weight. The same table also shows that there are significant differences between the concentrations of CPPU, where the concentration of 0.8 mg L^{-1} excelled on the rest of the concentrations.The highest value was recorded at 1.9908 mg.100 g^{-1} fresh weight, while the treatment with a concentration of 0 mg L^{-1} gave the lowest rate of 1.4925 mg.100 g^{-1} fresh weight.As for the effect of the interaction between the two experimental factors, the results in the same table indicated that there were significant differences The interaction treatment (0.8 CPPU × 7.5 PUT.) significantly excelled on the rest of the other interaction treatments .It gave the highest rate of 2.3367 mg.100 g-1 fresh weight, while the interaction treatment (PUT 0 × CPPU 0) gave the lowest rate of 1.2300 mg.100 g^{-1} fresh weight.

ISSN 2072-3857

Table (2): The effect of the growth regulators PUT and CPPU and their interaction on the percentage of carotene in the shoots (mg.100 g^{-1} fresh weight) of kumquat plants after four weeks of culture in vitro.

average CPPU	(PUT concentrations (mg.L-1)				CPPU concentrations mg.L-1
	7.5	5	2.5	0	
1.4925	1.3600	1.8133	1.5667	1.2300	0
1.8183	2.0833	1.4833	1.9667	1.7400	0.4
1.9908	2.3367	1.8833	1.6167	2.1267	0.8
	1.9267	1.7267	1.7167	1.6989	average PUT
0.01564 = PUT 0.01354=CPPU 0.02709 = interaction					LSD (0.05)

3- Total soluble sugars content of shoots (mg/g^{-1} dry weight)

The results presented in Table 3 showed that there were significant differences between the concentrations of PUT in shoots' content of total soluble sugars. The concentration of 7.5 mg L^{-1} was significantly higher, giving the highest rate of 10.0956 mg/g dry weight, compared to the lowest concentration of 0 mg L^{-1}, which gave 9.8278. mg/g dry weight. The results from the same table indicated that there were significant differences between the concentrations of CPPU added to the nutrient medium, which had a significant effect on shoots' content of total soluble sugars, where the concentration treatment of 0.8 mg L^{-1} achieved the highest percentage for this trait, amounting to 10.1792 mg/g dry weight, while the concentration treatment recorded 0 mg L^{-1}. The lowest percentage is 9.5783 mg/g dry weight. As for the effect of the interaction, the interaction treatment (0.8 CPPU + 7.5 PUT) was excelled, as it gave the highest content of total soluble sugars, amounting to 10.5700 mg/g dry weight, while the interaction (PUT 0 x CPPU 0) gave the lowest content, amounting to 9.2567 mg/g weight. dry.

Table (3): The effect of the growth regulators PUT and CPPU and their interaction on the soluble sugars content of shoots (mg/g dry weight) of kumquat plants after four weeks of culture in vitro.

average CPPU	(PUT concentrations (mg.L-1				CPPU concentrations mg.L-1
	7.5	5	2.5	0	
9.5783	9.4667	9.9133	9.6767	9.2567	0
9.9575	10.2500	9.5933	10.1367	9.8500	0.4
10.1792	10.5700	9.9800	9.7900	10.3767	0.8
	10.0956	9.8289	9.8678	9.8278	PUT average
0.02070 = PUT 0.01793=CPPU 0.03586 = interaction					LSD (0.05)

ISSN 2072-3857

4- *Auxin IAA content of shoots (µg.kg^{-1} fresh weight)*

The results of Table 4 show that increasing the levels of PUT in the MS nutrient medium had a significant effect on the average auxin content of shoots, as the concentration treatment of 7.5 mg L^{-1} recorded the highest rate of 13.5811 µg kg^{-1} fresh weight compared to the concentration treatment of 0 mg L^{-1}, which gave The lowest rate was 13.1489 µg kg^{-1} fresh weight.The results of the same table show a significant effect in increasing the content of shoots of the endogenous hormone indole acetic acid (IAA), where the content increased significantly with an increase in CPPU. Treatment 0.8 contained the highest rate of accumulation of acetic acid, which amounted to 13.7992 µg kg-1 fresh weight, while the lowest accumulation of this endogenous hormone was recorded in the treatment The concentration of 0 mg L^{-1} reached 12.6433 µg kg^{-1} fresh weight.The bi-interaction between the levels of PUT and CPPU had a significant effect on the content of shoots of indole acetic acid (IAA), where the interaction treatment (0.8 CPPU × CPPU 0) amounted to 12.0733 µg. kg^{-1} fresh weight.

Table (4): The effect of the growth regulators PUT and CPPU and their interaction on the auxin IAA content of shoots (µg kg^{-1} fresh weight) of kumquat plants after four weeks of culture in vitro.

average CPPU	(PUT concentrations (mg.L-1				CPPU concentrations mg.L-1
	7.5	5	2.5	0	
12.6433	12.3567	13.4200	12.7233	12.0733	0
13.3825	14.0233	12.5233	13.8133	13.1700	0.4
13.7992	14.3633	13.6933	12.9367	14.2033	0.8
	13.5811	13.2122	13.1578	13.1489	PUT average
0.02405 = PUT 0.02083 = CPPU 0.04166 = interaction					LSD (0.05)

Gibberellin content of shoots (µg.kg^{-1} fresh weight)

The results of Table 5 show that there are significant differences between the levels of PUT added to the MS nutrient media, as the 7.5 mg L^{-1} concentration treatment recorded the highest rate of 32.431 µg. kg^{-1} fresh weight compared to the concentration treatment of 0 mg L^{-1}, which gave the lowest rate of 31.546 µg. kg^{-1} fresh weight. The results of the same table also indicate that there are significant differences between the concentrations of CPPU added to the propagation medium in the amount of gibberellin accumulation in the shoots. In general, the rate of gibberellin accumulation increased at a concentration of 4 mg L^{-1} in the propagation medium, as the amount of gibberellin accumulated reached 32.249 µg. kg^{-1} fresh weight compared to the lowest amount at a concentration of 0 mg L^{-1},

ISSN 2072-3857

which amounted to 31.227 µg. kg^{-1} fresh weight. As for the results of the interaction between the two study factors regarding the gibberellin content of the shoots, the results of the same table indicate that there are significant differences between the intervention treatments, as the intervention treatment (0.8 CPPU Soft, while the interaction (PUT. 0 x CPPU 0) gave the lowest content, amounting to 30.597 µg. kg^{-1} fresh weight.

Table (5): The effect of the growth regulators PUT and CPPU and their interaction on the gibberellin content of shoots, µg.kg-1 fresh weight of kumquat plants after four weeks of culture in vitro.

average CPPU	mg.L-1 Levels PUT				CPPU concentrations mg.L-1
	7.5	5	2.5	0	
31.227	30.913	32.040	31.357	30.597	0
32.249	33.447	31.173	32.540	31.837	0.4
32.227	32.933	32.247	31.527	32.203	0.8
	32.431	31.820	31.808	31.546	average PUT
0.2869 = PUT			0.2484 =CPPU		
0.4969 = interaction					LSD (0.05)

Cytokinin content of shoots (µg.kg^{-1} fresh weight)

The results of Table 6 indicate that there are significant differences between the levels of PUT added to the nutrient medium in the average cytokinin content of the shoots, as the 7.5 mg L^{-1} concentration treatment gave the highest rate of 24.2344 µg kg^{-1} fresh weight compared to the 2.5 treatment, which gave the lowest rate of 23.5111. µg. kg^{-1} fresh weight. It is indicated from the same table that the amount of cytokinin accumulation increases with increasing CPPU in the multiplication medium, as it appeared that there were significant differences between the CPPU added to the multiplication medium in the cytokinin content of the shoots, with the control treatment 0.8 mg L^{-1} being excelled on the rest of the treatments in the content of cytokinin, which recorded 24.4975 µg. kg-1 fresh weight compared to the control treatment 0 mg L^{-1}, which gave the lowest content of 22.8333 µg kg^{-1} fresh weight.The results of the interaction between the two study agents in the same table show that there are significant differences between the interaction treatments, where the interaction treatment (0.8 CPPU × 7.5 PUT.) was significantly excelled on the rest of the interactions, which gave 25.3733 µg. kg-1 fresh weight, noting that the lowest values recorded during the intervention treatment (PUT. 0 x CPPU 0) amounted to 22.1233 µg kg-1 fresh weight.

ISSN 2072-3857

Table (6): The effect of the growth regulators PUT and CPPU and their interaction on the cytokinin µg content of shoots. Kg^{-1} fresh weight of kumquat plants after four weeks of culture in vitro

average CPPU	(PUT concentrations (mg.L-1				CPPU concentrations mg.L-1
	7.5	5	2.5	0	
22.8333	22.4333	23.9467	22.8300	22.1233	0
23.9150	24.8967	22.6933	24.5333	23.5367	0.4
24.4975	25.3733	24.2100	23.1700	25.2367	0.8
	24.2344	23.6167	23.5111	23.6322	PUT average
0.02972= PUT 0.02574=CPPU 0.05148 = interaction					LSD (0.05)

Discussion

This may be due to the distinct role of Putrescine in the process of cell division and thus increasing the number of cells in biologically active areas [4] . The reason is attributed to the role of Putrescine in increasing the accumulation of cytokinin in plant tissues, and this is supported by the results of Table No. (15), as cytokinin works to reduce the replication of And the growth of branches by breaking the apical dominance.

What is the positive response in the average number of leaves, Table (3), and the rate of increase in the length of branches, Table (2) This is due to the stimulating action of cytokinins in urging cells to divide and differentiate, and this results in the differentiation of the transplanted tissues and the density of the shoots. Many researchers have indicated the role that cytokinins play in the concentrations. Suitability in tissue culture, including [6,7,11] .

The study of adding cytokinins agreed with the findings of [1,13] which had a significant effect in increasing the number of leaves and the length of branches of the Swingle citrumelo rootstock relative to the other concentrations.

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ISSN 2072-3857

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